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Sleep Disturbance and Altered Expression of Circadian Clock Genes in Patients With Sudden Sensorineural Hearing Loss

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Abstract: The cause of sudden sensorineural hearing loss (SSNHL) remains unclear and therefore it is often considered as idiopathic. Sleep disturbance has been linked to SSNHL and circadian rhythm disruption, but the link between circadian rhythm disruption and SSNHL has never been investigated.

In this study, we surveyed the sleep quality of 38 patients with SSNHL using a simple insomnia sleep questionnaire. The expression of circadian clock genes in peripheral blood (PB) leukocytes from 38 patients with SSNHL and 71 healthy subjects was accessed using real-time quantitative reverse transcriptase-polymerase chain reaction and validated using immunocytochemical staining.

We found that 61.8% of patients with SSNHL suffered from insomnia before the insult of hearing loss. Besides, significantly decreased expression of *PER1*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, and *CKIε* was found in PB leukocytes of patients with SSNHL when compared with healthy subjects. SSNHL patients with vertigo had significantly lower expression of *CRY1* and *CKIε* than patients without vertigo symptoms. Our results imply the association of sleep disturbance and disrupted circadian rhythm in SSNHL.

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Abbreviations: ΔCt = threshold cycle difference, ACTB = β-actin, HPA = hypothalamic–pituitary–adrenal, HRP-DAB = horseradish peroxidase-diaminobenzidine, ISQ = Insomnia Sleep Questionnaire, MSQ = Mini Sleep Questionnaire, PB = peripheral blood, PBS = phosphate-buffered saline, PTA = pure-tone average, qRT-PCR = real-time quantitative reverse transcriptase-polymerase chain reaction, SSNHL = sudden sensorineural hearing loss.

INTRODUCTION

Sudden sensorineural hearing loss (SSNHL) is commonly defined as a more than 30 dB SSNHL in 3 contiguous frequencies over a time course of less than 3 days. The incidence of SSNHL ranges from 5 to 20 per 100,000 subjects per year. The causes of SSNHL are still hard to be found and therefore are often thought as "idiopathic." The treatment modalities of SSNHL is not largely changed and not improved in the past decades due to the mysteries of exact etiologies and risk factors of SSNHL. Although alteration of genes related to prothrombotic[1] and inflammatory[2] factors has been found in some sudden hearing loss patients, it is still not clear which environmental factor could predispose patients to undergo sudden hearing loss.

Circadian rhythm is present in almost all eukaryotes with a 24-hour cycle. Daily rhythmic changes are found in many physiological processes, including sleep, appetite, hormone level, metabolism, and gene expression.[3] The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the internal clock controlling various physiological systems to a cycle of 24 hours. In addition to central pacemaker, peripheral organs, such as liver, heart, kidney, and peripheral blood (PB) leukocytes, also contain circadian oscillators.[4] In the ear, diurnal changes of otoacoustic emissions have been reported,[5–7] suggesting that outer hair cells of the cochlea may also be synchronized with the biological circadian rhythm.

There are at least 9 core circadian clock genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *CK1ε*, *BMAL1*, and *TIM*) that regulate central and peripheral circadian oscillators using transcriptional–translational feedback loops.[3,8,9] Disruption of circadian rhythm or altered circadian clock genes are associated with increased risks of depression,[10] diabetes,[11] and cancer development.[12–15] Our previous study also demonstrated that the daily pattern of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *CKIε* expression level peaked at 8:00 AM and *BMAL1* peaked at 8:00 PM in PB total leukocytes of healthy individuals, but the daily pattern expression of these 7 genes was disrupted in newly diagnosed preimatinib mesylate-treated and blast crisis-phase patients with chronic myeloid leukemia.[15]

In a previous report, short sleep duration was found to be as a risk factor for idiopathic SSNHL.[16] An association between obstructive sleep apnea and SSNHL has also been found recently.[17] However, the relationship between sleep disorders and circadian rhythm in patients with SSNHL has never been reported. Whether sleep disturbance or circadian rhythm disruption are vulnerable factors for SSNHL remain elusive. Therefore, we designed a questionnaire to survey if sleep disturbance occurred before the hearing loss attack and to investigate the expression of the 9 circadian clock genes of

PB leukocytes by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in patients with SSNHL.

MATERIALS AND METHODS

Subjects

This study enrolled 38 patients with SSNHL who were admitted to Kaohsiung Chang Gung Memorial Hospital, Taiwan, with the diagnosis of idiopathic SSNHL from October 2010 throughout December 2013. SSNHL was defined as a hearing loss of 30 dB or more over at least 3 contiguous frequencies within 72 hours. We excluded the SSNHL cases with known origins, including those whose hearing loss was caused by retro-cochlear lesions as detected by acoustic brainstem response and magnetic resonance imaging, and whose SSNHL was caused by infectious or autoimmune diseases as detected by laboratory examinations. Patients who suffered from bilateral SSNHL or ever received steroid therapy previously were also excluded. Clinical parameters including pure-tone average (PTA) of 500, 1000, 2000, and 4000 Hz and associate symptom of vertigo in 38 SSNHL patients during admission were recorded. Also recruited were 71 age-matched healthy subjects who did not have cochlea-vestibular disorders. Table 1 presents the clinical characteristics of patients with SSNHL and controls. Informed consent was obtained from all patients and healthy subjects before PB collection. This study was reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Sleep Questionnaire

We used an Insomnia Sleep Questionnaire (ISQ) (Table 2) to evaluate if sleep disturbance occurred before the episode of SSNHL. This ISQ is part of the Mini Sleep Questionnaire (MSQ)[18,19] which is a validated sleep quality questionnaire. The response scale of ISQ ranges from 1 (No) to 2 (Yes). A total score of 4 is considered representative of good sleep quality and a score of more than 4 is considered as poor sleep quality and regarded as "insomnia."[20] During admission, patients with SSNHL were asked to fill out the questionnaire regarding the sleeping pattern of the recent 1 week before the episode of hearing loss.

TABLE 1. Characteristics of SSNHL Patients and Controls

Parameter	SSNHL (n = 38)	Control (n = 71)	P-Value
Age	51.39 ± 18.24*	50.25 ± 13.27	0.74
Sex			0.42
Male	21	42	
Female	17	29	
Hearing severity			
Mild HL (<55 dB)	3		
Moderate HL (55–70 dB)	8		
Severe HL (71–90 dB)	5		
Profound HL (>90 dB)	22		
Vertigo			
Yes	17		
No	21		

HL = hearing loss, SSNHL = sudden sensorineural hearing loss.
* Data are presented as mean ± standard deviation.

TABLE 2. Insomnia Sleep Questionnaire (ISQ)

Insomnia Sleep Questionnaire Item	No	Yes
Difficulty falling asleep	1	2
Waking up too early	1	2
Mid-sleep awakening	1	2
Hypnotic medication use	1	2
Total score:		
ISQ scores of SSNHL patients*		
Total score = 4 (good sleep quality)	n = 13 (38.2%)	
Total score > 4 (insomnia)	n = 21 (61.8%)	

ISQ = Insomnia Sleep Questionnaire, SSNHL = sudden sensorineural hearing loss.
* Sleep questionnaires were obtained from 34 out of the 38 patients with SSNHL.

qRT-PCR Analysis of Circadian Clock Genes

PB samples were obtained between 8:00 and 9:00 AM from patients with SSNHL before steroid treatment and from healthy controls because our previous study showed that the expression level of most circadian clock genes in PB leukocytes peaked at 8:00 AM.[15] TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA from the PB leukocytes and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to generate cDNA according to the manufacture's protocols. The specific forward and reverse primers and MGB TaqMan® probes for the 9 circadian clock genes and *ACTB* (β-actin) gene for qRT-PCR analysis are as described previously.[14] For internal control of the RNA, expression of *ACTB* gene was also examined by qRT-PCR to normalize the expression of circadian clock genes. qRT-PCR was carried out in a 10-μL final volume containing 25 ng cDNA, 400 nM each primer, 200 nM probe, and 5 μL 2× TaqMan® Universal PCR Master Mix (Applied Biosystems) in an ABI 7500 Fast Real-Time System (Applied Biosystems). The PCR cycling parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 minute. The expression levels of the circadian clock genes were normalized to the internal control *ACTB* to obtain the relative threshold cycle (ΔCt). The relative expression in patients with SSNHL compared with control is calculated by ΔΔCt [ΔCt (SSNHL-*ACTB*)-ΔCt (Control−*ACTB*)]. The fold change in patients with SSNHL compared with control is then calculated by $2^{-\Delta\Delta Ct}$ method.

Immunocytochemistry

Imunocytochemical staining was performed on PB total leukocyte samples of patients with SSNHL and healthy controls. Isolated PB leukocytes (5×10^5) were placed onto glass slides by cytocentrifuged at 500 rpm for 5 minutes. The slides were allowed to air-dry for 10 to15 minutes before fixing in 1% formaldehyde/phosphate-buffered saline (PBS) and blocking for nonspecific binding with 10% bovine serum albumin/PBS. Samples were first incubated with polyclonal antibodies against PER1 and CRY1 (Abcam, Inc., Massachusetts) at 1:200 dilutions for 1 hour then incubated with biotinylated goat anti-rabbit antibodies for 30 minutes. A horseradish peroxidase-diaminobenzidine (HRP-DAB) staining kit (Abcam, Inc.) was used to visualize the specific binding of the secondary antibodies to the primary antibodies. After staining, the cells were mounted with glass slides, cover-slipped, and examined using a Zeiss microscope (Zeiss, Gottingen, Germany).
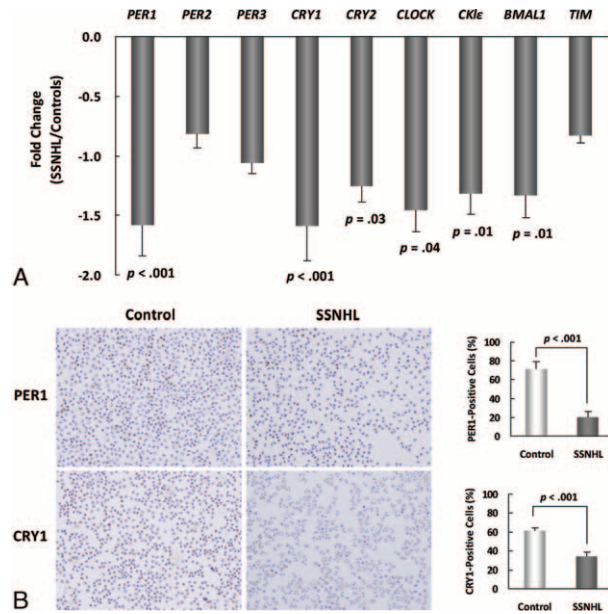
FIGURE 1. The expression levels of the 9 circadian clock genes in patients with SSNHL and controls determined by qRT-PCR and confirmed by immunocytochemical staining. (A) Expression of the 9 circadian clock genes in PB leukocytes from 38 patients with SSNHL compared with 71 healthy controls. The *y*-axis represents the fold change of mRNA expression level in patients with SSNHL compared with controls. The amount of circadian clock gene was normalized to the endogenous reference gene *ACTB* to obtain the relative threshold cycle (ΔCt). The normalized circadian clock gene expression (ΔCt) of patients with SSNHL was then related to the ΔCt of controls for their relative expression levels. The relative expression in patients with SSNHL compared with control is calculated by ΔΔCt [ΔCt (SSNHL - *ACTB*) - ΔCt (normal – *ACTB*)]. The fold change of mRNA expression in patients with SSNHL compared with control is calculated by designating the expression in controls as 1 then calibrated to the mRNA expression in patients with SSNHL by $2^{-\Delta\Delta Ct}$ calculation. The *P*-value indicated is the statistical significance evaluated between patients with SSNHL (n = 38) and controls (n = 71) using ΔCt values. All reactions were run in duplicate. (B) Immunocytochemical staining for PER1 and CRY1 proteins. In PB leukocytes from controls, most cells are positively stained (brown) for PER1 and CRY1 antibodies, but only few cells of the patients with SSNHL were stained. Antibodies' staining were detected using peroxidase with diaminobenzidine (DAB) substrate. Cells were counterstained with hematoxylin–eosin. The original magnification is 200× and representative microscopic fields are shown. Percentages of cells positively stained for PER1 or CRY1 antibodies were calculated from 3 PB samples of patients with SSNHL and 3 PB samples of controls. The difference between SSNHL group and control group was analyzed using a paired *t* test.

Statistical Analysis

The SPSS software for Windows (version 13.0, SPSS, Inc., Chicago, IL) was used for all the statistical analyses of this study. The values of ΔCt were used for the statistical analysis of gene expression. Chi-square test was used to compare the categorical variables and the differences in each circadian clock gene expression between 2 groups were evaluated using the independent *t* test or Mann–Whitney *U* test. A *P*-value <0.05 was considered statistically significant. All statistical tests were 2-sided.

RESULTS

Sleep Questionnaires of Patients With SSNHL

Sleep questionnaires were obtained from 34 out of the 38 patients with SSNHL. The 4 missing questionnaires was due to unwillingness to answer (1 cases), failure to remember the sleep pattern (1 cases), and noncooperation of the patients (2 cases). As summarized from the questionnaires, the most common complaints of sleep symptoms were waking up too early (38.2%, 13 in 34 cases) and mid-sleep awakening (38.2%, 13 in 34 cases). The less common complaints were difficulty falling asleep (11.8%, 4 in 34 cases) and hypnotic medication use (11.8%, 4 in 34 cases). When defining insomnia as ISQ score > 4, 61.8% (21 in 34 cases) of patients with SSNHL suffered from insomnia before the episode of sudden hearing loss.

Expression of Circadian Clock Genes in PB Leukocytes of Patients With SSNHL and Controls Using qRT-PCR

Figure 1A demonstrates the altered expression of the 9 circadian clock genes in patients with SSNHL compared with normal controls by qRT-PCR. Of the 9 circadian clock genes, the expression of *PER1*, *CRY1*, *CRY2*, *CLOCK*, *Ck1ε*, and *BMAL1* are significantly decreased in patients with SSNHL compared with controls (*P* < 0.05). Among the 6 downregulated genes, *CRY1* and *PER1* were the 2 most downregulated genes. We also conducted an immunocytochemical staining of PB leukocytes to validate the protein expression of PER1 and CRY1, which were the 2 most downregulated genes. As shown in Figure 1B, most cells of the control were positively stained by the PER1 and CRY1 antibodies. However, only few cells of the patients with SSNHL were stained. The immunocytochemical findings confirmed the transcript results of gene expression acquired from qRT-PCR analysis.
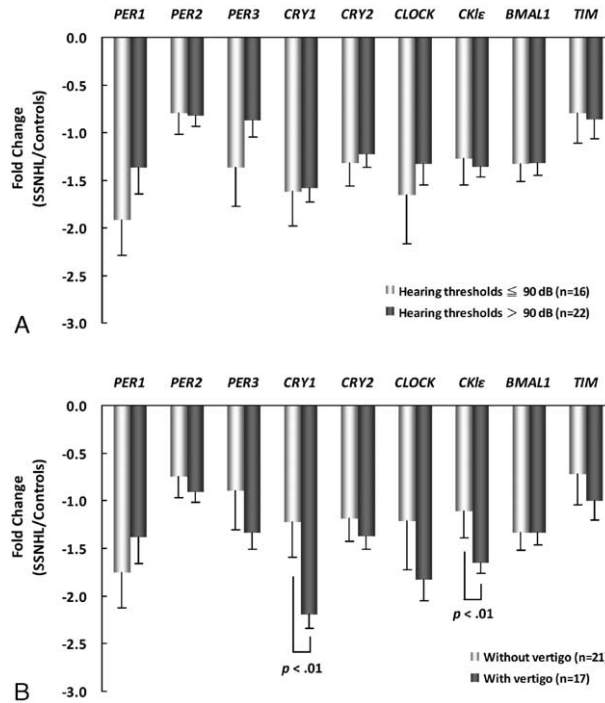

FIGURE 2. Severity of hearing loss, vertigo, and circadian clock gene expression in patients with SSNHL. (A) There is no significant difference of the expression of the 9 circadian clock genes in PB leukocytes between SSNHL patients with profound hearing loss (hearing thresholds > 90 dB) and nonprofound hearing loss (hearing threshold ≤ 90 dB). The amount of circadian clock gene was normalized to the endogenous reference gene *ACTB* to obtain the relative threshold cycle (ΔCt). The normalized circadian clock gene expression (ΔCt) of SSNHL patients with profound hearing loss or nonprofound hearing loss was then related to the ΔCt of controls for their relative expression levels. The relative expression in SSNHL patients with profound hearing loss compared with control is calculated by ΔΔCt [ΔCt (SSNHL profound hearing loss – *ACTB*) - ΔCt (Control - *ACTB*)]. The relative expression in SSNHL patients with nonprofound hearing loss compared with control is calculated by ΔΔCt [ΔCt (SSNHL nonprofound hearing loss – *ACTB*) – ΔCt (Control – *ACTB*)]. The value of mRNA expression in controls is designated 1, and the level of mRNA expression in profound hearing loss group or nonprofound hearing loss group is calibrated to obtain the folds changed by $2^{-\Delta\Delta Ct}$ calculation. All reactions were run in duplicate. The *P*-value indicated is the statistical significance evaluated between profound hearing loss group (n = 22) and nonprofound hearing loss group (n = 16). (B) The expression of *CRY1* and *CKIε* genes in PB leukocytes is significantly lower in SSNHL patients with symptom of vertigo than those without vertigo. The amount of circadian clock gene was normalized to the endogenous reference gene *ACTB* to obtain the relative threshold cycle (ΔCt). The normalized circadian clock gene expression (ΔCt) of SSNHL patients with symptom of vertigo or without vertigo was then related to the ΔCt of controls for their relative expression levels. The relative expression in SSNHL patients with symptom of vertigo compared with control is calculated by ΔΔCt [ΔΔCt = ΔCt (SSNHL with vertigo-*ACTB*)-ΔCt (Control-*ACTB*)]. The relative expression in SSNHL patients without vertigo compared with control is calculated by ΔΔCt [ΔCt (SSNHL without vertigo-*ACTB*)-ΔCt (Control-*ACTB*)]. The value of mRNA expression in controls is designated 1, and the level of mRNA expression in vertigo group or without vertigo group is calibrated to obtain the folds changed by $2^{-\Delta\Delta Ct}$ calculation. All reactions were run in duplicate. The *P*-value indicated is the statistical significance evaluated between vertigo group (n = 17) and without vertigo group (n = 21).

Correlations Between Expression of Circadian Clock Genes in PB Leukocytes and Hearing Severity or Symptoms of Vertigo in Patients With SSNHL

Correlation analysis of the 9 circadian clock genes expression was performed in 22 patients with profound SSNHL (hearing threshold > 90 dB HL in affected ear) and 16 patients with nonprofound SSNHL (hearing threshold ≤ 90 dB HL in affected ear). However, no significant differences were observed between the 2 groups (*P* > 0.05; Figure 2A). When dividing patients with SSNHL into vertigo (n = 17) and non-vertigo (n = 21) groups according to the associated symptom of vertigo during the episode of hearing loss, significantly lower expression of *CRY1* (*P* = 0.002) and *Ck1ε* (*P* = 0.008) was found in patients with SSNHL combined with vertigo symptoms compared with those without vertigo (Figure 2B).

Expression of Circadian Clock Genes in PB Leukocytes of SSNHL Patients With and Without Sleep Disturbance

In SSNHL patients with precipitating insomnia before the attack of hearing loss (ISQ > 4), the expression of *PER1* and *CRY2* in PB leukocytes were significantly lower than those without insomnia (ISQ = 4) (Figure 3). The results suggest that altered circadian clock genes expression in PB is coordinated with the insomnia symptoms subjectively described by patients with SSNHL.

DISCUSSION

SSNHL is an acute debilitating form of SSNHL and the exact etiologies of most patients were failed to be obtained. In our clinic, most patients with SSNHL could not describe special precipitating symptoms. However, some of them often
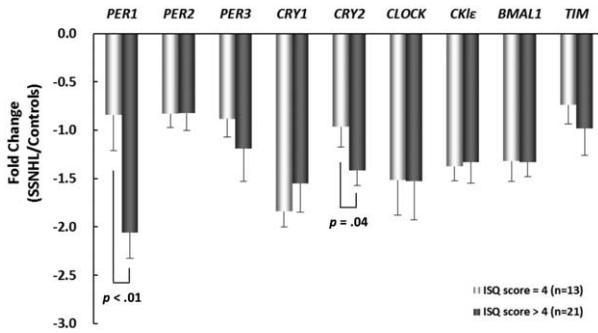
FIGURE 3. The expression of *PER1* and *CRY2* gene in PB leukocytes is significantly lower in SSNHL patients with insomnia (ISQ score > 4) than those without insomnia (ISQ score = 4). The amount of circadian clock gene was normalized to the endogenous reference gene *ACTB* to obtain the relative threshold cycle (ΔCt). The normalized circadian clock gene expression (ΔCt) of SSNHL patients with insomnia or without insomnia was then related to the ΔCt of controls for their relative expression levels. The relative expression in SSNHL patients with insomnia compared with control is calculated by ΔΔCt [ΔΔCt = ΔCt (SSNHL with insomnia-*ACTB*)-ΔCt (Control-*ACTB*)]. The relative expression in SSNHL patients without insomnia compared with control is calculated by ΔΔCt [ΔCt (SSNHL without insomnia-*ACTB*)-ΔCt (Control-*ACTB*)]. The value of mRNA expression in controls is designated 1, and the level of mRNA expression in insomnia group or without insomnia group is calibrated to obtain the folds changed in patients with SSNHL by $2^{-\Delta\Delta Ct}$ calculation. All reactions were run in duplicate. The *P*-value indicated is the statistical significance evaluated between insomnia group (n = 21) and without insomnia group (n = 13).

described interrupted sleep duration or insomnia days before the attack of hearing loss. Because insomnia is one of the common symptoms in circadian rhythm sleep disorders,[21] it is interesting to know if these patients with SSNHL had disrupted circadian rhythm. This is the first study that investigated the circadian rhythm in patients with SSNHL and altered expression of circadian clock genes was found in patients with SSNHL.

Sleep disturbance is widely found in patients with tinnitus,[22–24] but the relationship of sleep disorder and cochlea-vestibular diseases is still poorly understood. Hearing impairment has been noted in cases of sleep disturbance.[23,25] A link has also been found between idiopathic dizziness and sleep apnea.[26] However, these studies only investigated the sleep quality of subjective that relied on self-reported questionnaires but did not analyze the data objectively. In a case–control study, SSNHL has been found in association with obstructive sleep apnea,[17] but the effect of sleep quality on the mechanism of SSNHL is less understood. Our study not only provided evidence for the existence of sleep disturbance in patients with SSNHL by a validated questionnaire, but also found the decreased expression the circadian clock genes in patients with SSNHL. The results of circadian clock gene expression in PB are coordinated with the sleep questionnaires done by patients with SSNHL. Because the circadian genes expression levels in blood sample have been suggested as appropriated markers for estimating an individual's circadian rhythm,[27] our findings imply that disrupted circadian rhythm may associate with the episode of SSNHL.

Another interesting finding is the significantly lower expression of some circadian clock genes in SSNHL patients with symptoms of vertigo than those without vertigo. Because

SSNHL patients with symptom of vertigo often present as unilateral vestibular hypofunction,[28,29] that means this group of patients have more severe involvement of inflammation over cochlear-vestibular organ. In contrast to patients with profound SSNHL whose expression of circadian clock genes is not more downregulated than those with nonprofound SSNHL, the associated symptoms of vertigo in SSNHL may be more obviously affected by disrupted circadian rhythm. These results hint that circadian rhythm dysregulation could more markedly damage vestibular system in SSNHL.

The expression of circadian clock genes dictates the temporal regulation of clock-output/functional rhythm in brain regions and peripheral tissues.[9,30] Dysregulation of circadian parameters are frequently found in central nervous system disorders.[31] In previous literatures, decreased expression of some circadian clock genes has been found in PB leukocytes of patients with Parkinson's disease[32] and bipolar disorder.[33] In addition to central clock, circadian rhythm in peripheral tissues could also controlled by the SCN of the anterior hypothalamus via hormonal and autonomic signal.[4] One good example is the daily corticosteroid peak in mammals achieved by hypothalamic–pituitary–adrenal (HPA) axis. Because corticosteroid is essential in keeping homeostatic balance of inner ear,[34] disrupted circadian rhythm may cause dysregulation of circadian corticosteroid peak in the body, and in turn affect the function of inner ear. This may account for our findings that altered expression of circadian clock genes occurred during the attack of SSNHL in patients.

Another function of circadian rhythm is the modulation of stress responses. Perturbations of circadian rhythms by external or internal stressors may negatively impact health by impairing immune function.[35,36] Because stress response is hypothesized as one of the possible etiologies of SSNHL in recent years,[37,38] the association of disrupted circadian rhythm and the occurrence of SSNHL could be postulated. A systemic viral illness, inflammatory disorder, and mental or metabolic stress may induce the innate and adaptive immunity, which will result in the activation of NF-κB in the cochlea. Once the patients suffered from disrupted circadian rhythm due to sleep disturbance, the circadian clock in immune cells is also disturbed. The preceding results may be the abnormal persisted production of inflammatory cytokines. Our hypothesis could explain why some patients with SSNHL described symptoms of upper respiratory infection and insomnia at the same time before the episode of hearing loss. When patients suffered from "common cold," sleep disturbance may disturbed the circadian rhythm in immune system, and strengthen the inflammatory stress response in the cochlea, which then cause a "second hit" upon the inner ear. However, the mechanisms underlying the association between disrupted circadian rhythm and SSNHL still require further investigation.

The limitation of our study is that when we conducted the sleep questionnaires to investigate the sleep quality in patients with SSNHL, they may not have remembered correctly their sleep patterns 1 week before. This subjective factor would affect the reliability of sleep evaluation. This and some technical issues of obtaining the sleep questionnaires from the control population are important considerations for our future experimental designs. It may also imply that the examination of the circadian clock genes from the PB will be a better reflection of the objective evaluation of circadian rhythm disruption and sleep quality than questionnaires. Another limitation of this study is that we could only measure the expression of genes during the daytime. However, the significant downexpression of

several genes from the same time point in patients with SSNHL compared with control also implies the link between the disruption of circadian rhythm and SSNHL.

Because the evidences of classical viral or vascular etiologies in SSNHL cannot be well demonstrated, it is crucial to recognize other factors that may predispose patients to suffer from this disease. Our results of this study present the association of sleep disturbance and altered circadian clock genes with SSNHL. Decreased expression of circadian clock genes, *CRY1* and *CK1ε*, in PB leukocytes is more obviously found in SSNHL patients with vertigo. Further studies are needed to elucidate the effect of disrupted circadian rhythm on cochlea-vestibular function and pathogenesis of SSNHL.

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